The semaphorin 3A receptor may directly regulate the activity of small GTPases

Beate Rohm, Belquis Rahim, Bedriska Kleiber, Iiris Hovatta, Andreas W. Püschel*

Molecular Neurogenetics Laboratory, Department of Neurochemistry, Max-Planck-Institute for Brain Research, Deutschordenstr. 46, D-60528 Frankfurt, Germany

Received 1 November 2000; accepted 8 November 2000

First published online 16 November 2000

Edited by Jesus Avila

Abstract The axon guidance signal semaphorin 3A induces the rapid collapse of growth cones by activating a receptor complex that contains neuropilin-1 as the ligand-binding and a plexin as the signal-transducing subunit. Here we show that plexins bind Rho-like GTPases and may directly regulate their activity. The cytoplasmic domain of plexins shows sequence similarity to GTPase activating proteins (GAPs) and mutation of two arginines that correspond to the catalytic residues of Ras GAPs inactivates plexin-A1. Our data suggest that plexins may be integral membrane proteins with an intrinsic GAP activity that is essential for their ability to induce growth cone collapse.

Key words: Plexin; Neuropilin; Rac; Collapse; Axon guidance

1. Introduction

During the development of neuronal connections, chemotropic signals play an essential role in the guidance of axons to their target areas. The semaphorins are one of several protein families that can act as chemorepellents and perform important functions in the developing nervous system of both vertebrates and invertebrates [1–3]. Especially semaphorin 3A (Sema3A) exerts powerful effects on a wide variety of axons [4–10]. The exposition of growth cones to Sema3A induces a dramatic redistribution of actin filaments ultimately resulting in their collapse [11]. However, the donwstream signal transduction events that translate the binding of axonal guidance signals to their receptors into structural changes of the cytoskeleton are largely unknown.

Sema3A activates a receptor complex that contains neuropilin-1 (Nrp-1) as the ligand-binding subunit and a plexin as the signal-transducing subunit [12–15]. The plexins belong to a superfamily of semaphorin domain containing proteins that also includes the semaphorins and c-Met related receptor-type tyrosine kinases [16–18]. Nine plexins have been identified so far in mammals that fall into four distinct classes (classes A–D) [12,13,15]. Closely related sequences have been found also in *Drosophila melanogaster* and *Caenorhabditis elegans* [18]. While class 3 semaphorins do not bind to A-type plexins and require the neuropilins as co-receptors, *Drosophila* plexin-A, -B1 and -C1 can directly interact with Sema1a, Sema4B and Sema7A, respectively [12,13,15,18,19].

The activation of the Sema3A receptor leads to the depolymerization of F-actin by an unknown mechanism. The block of Sema3A-induced growth cone collapse by dominant-negative N17Rac suggests the involvement of small Rho-like GTPases [20–22]. The cytoplasmic domain is highly conserved between different plexins and shows sequence similarity to Ras GTPase activating proteins (GAPs). Here we demonstrate that this domain includes two essential conserved arginine residues that correspond to the invariant catalytic residues of Ras GAPs. Their mutation abolishes the ability of plexin-A1 to induce cell collapse in response to Sema3A in a heterologous system. In addition, we demonstrate differential binding of the Rho-like GTPases Rnd1 and Rac1 to plexin-A1 and -B1, respectively. Our data suggest that plexins may possess intrinsic GAP activity that is essential for their ability to induce growth cone collapse and thereby directly regulate the activity of small GTPases.

2. Materials and methods

2.1. Vectors

The expression vectors for AP-Sema3AP1b, HANrp-1, VSVPlexin-A1 and VSVPlexin-B1 have been described before [15]. Point mutations (R1429,1430A and R1746A) were introduced into pBKVSVPlexin-A1 using the QuickChange mutagenesis kit (Stratagene) according to the manufacturer's recommendations. The coding sequences for murine Rac1, RhoA and human Rnd1 were amplified from mouse E12.5 spinal cord or human fetal brain (Stratagene) cDNA and cloned into the pGEX4T2 vector (AmershamPharmacia).

2.2. Glutathione S-transferase (GST) pull-down

GST-fusion proteins were expressed in *Escherichia coli* M15 (Qiagen) and bacterial extracts prepared in BLB (phosphate-buffered saline (PBS) supplemented with 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), complete protease inhibitor cocktail (Roche), 10% glycerol) using a French press. 293T cells were transfected by calcium phosphate co-precipitation as described previously [10]. After 48–72 h of culture, cells were lyzed in TLB (1% Triton X-100, 50 mM Tris/HCl pH 7.4, 1 mM DTT, 1.5 mM $MgCl_2$, 4 mM EDTA, 10% glycerol and complete protease inhibitor cocktail (Roche)) for 30 min. Cleared lysates were incubated for 2 h with glutathione Sepharose 4B bound GST-fusion proteins that were preloaded with GTPγS as described by Brill et al. [23]. After three washes with TWB (0.1% Triton X-100, 50 mM Tris/HCl pH 7.4, 1 mM DTT, 1.5 mM $MgCl_2$, 5 mM EDTA, 10% glycerol) bound proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer, separated by SDS–PAGE, blotted onto PVDF membranes (AmershamPharmacia) and revealed with anti-VSV (Roche) or anti-GST antibodies (AmershamPharmacia) following the manufacturer's instructions.

*Corresponding author. Fax: (49)-69-96769 441.
E-mail: pueschel@mpih-frankfurt.mpg.de

0014-5793/00/$20.00
PII: S0014-5793(00)02240-7

2.3. Binding and COS collapse assay

Binding assays with AP-Sema3AP1b and transfected 293T cells were done as described [15]. For the collapse assay COS-7 cells were transfected using the Fugene 6 reagent (Roche). Two days after transfection, cells were incubated with APSema3AP1b for 1 h at 37°C and fixed with 3.7% formaldehyde in PBS. Endogenous phosphatases were inactivated for 45 min at 65°C and bound AP-fusion protein revealed as described. The number of collapsed and uncollapsed cells was determined following the criteria of Takahashi et al. [12].

3. Results

Within the cytoplasmic domain of plexin-A1 two subdomains, separated by 219 amino acids, show sequence homology to Ras GAPs (Fig. 1). These sequences are 30% identical and 48% similar to SynGAP or R-RasGAP, respectively, a degree of homology that is comparable to that between the Ras GAPs p120 RasGAP and SynGAP. The homologous regions include two invariant arginines (R1430 and R1746 in plexin-A1, single letter code; Fig. 1) that correspond to residues essential for the catalytic function of GAPs [24–26].

One prerequisite for the function of plexins as GAPs is their ability to interact with GTP-binding proteins. A role in regulating the actin cytoskeleton is firmly established for Rac1 and RhoA [27] Therefore we used a pull-down assay with bacterially expressed GST-fusion proteins of Rac1, RhoA and the Rho-like GTPase Rnd1 that has been shown to promote the dizassembly of actin filaments [28]. The GST-fusion proteins were immobilized on glutathione Sepharose beads, preloaded with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and incubated with a lysate of 293T cells that had been transfected with expression vectors for plexin-A1 or -B1. Proteins bound to the GST-fusion proteins were eluted and analyzed by Western blot. Rnd1 bound to plexin-A1 and, to a lesser extent, also to plexin-B1 while Rac1 interacted only with plexin-B1 (Fig. 2A). No interaction with plexins was detectable for RhoA, GST or when the cytoplasmic domain of plexin-A1 was deleted, confirming the specificity of the assay (Fig. 2A,B). Rnd1 and Rac1 differed in the GTP dependence of their association with plexins (Fig. 2C). The binding of Rnd1 to plexin-A1 was detectable when Rnd1 was pre-incubated with either GTPγS or GDP. In contrast, only GTP-Rac1 interacted with plexin-B1.

In order to investigate if plexin-A1 might indeed act as a GAP, we tested if the conserved arginine residues in the cytoplasmic domain of plexin-A1 are required for its function and mutated R1429,1430 and R1746 to alanine separately (R1: R1429,1430A, R2: R1746A) or in combination (R12: R1429,1430, 1746A). These mutations did not affect the interaction of plexin-A1 with Nrp-1 and its ligand Sema3A as the binding properties of the double mutant were identical to that of wild-type plexin-A1. Co-expression of plexin-A1 or -A1R12 with Nrp-1 in 293T cells resulted in an increase in the number of binding sites for Sema3A compared to expression of Nrp-1 alone as has been described before for plexin-A1 [15] (Fig. 3A). Plexin-A1 mutants and plexin-A1 were expressed at comparable levels on the cell surface of 293T and COS-7 cells (Fig. 4A and data not shown) and plexin-A1R12 bound Rnd1 like wild-type Plexin-A1 (Fig. 3B).

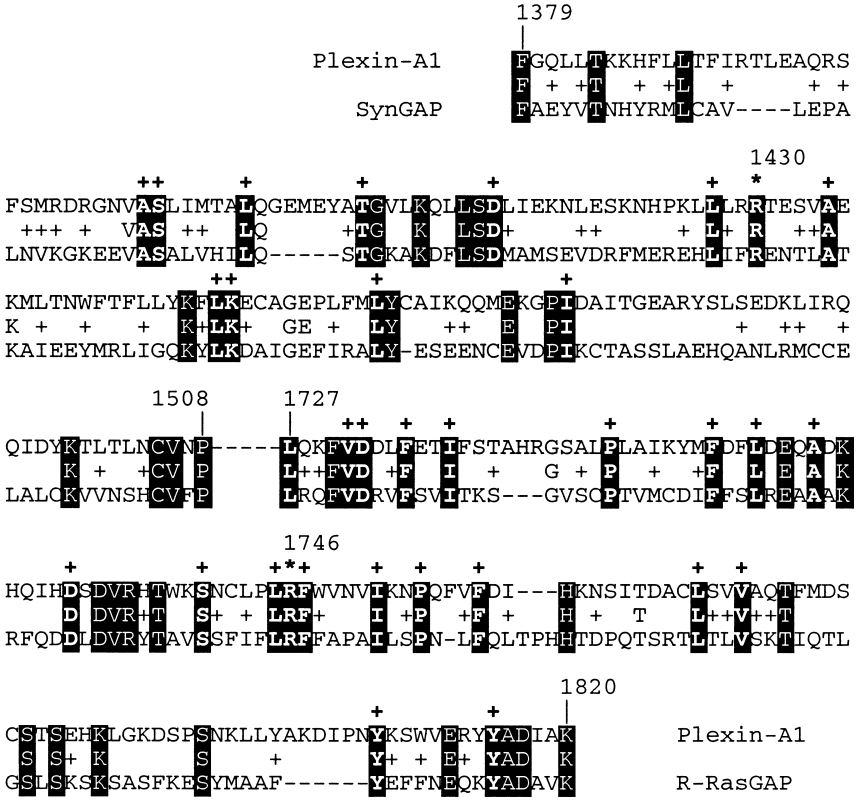

Fig. 1. The cytoplasmic domain of plexin-A1 shows sequence similarity to Ras GAPs. Alignments of the amino acid sequence of plexin-A1 (position 1379 to 1820, GenBank accession number D86948) with partial sequences of SynGAP (AF048976) or R-RasGAP (U30857) are shown. Residues conserved between all plexins and these proteins are indicated by + signs and the conserved arginine residues R1430 and R1746 by asterisks above the plexin-A1 sequence.

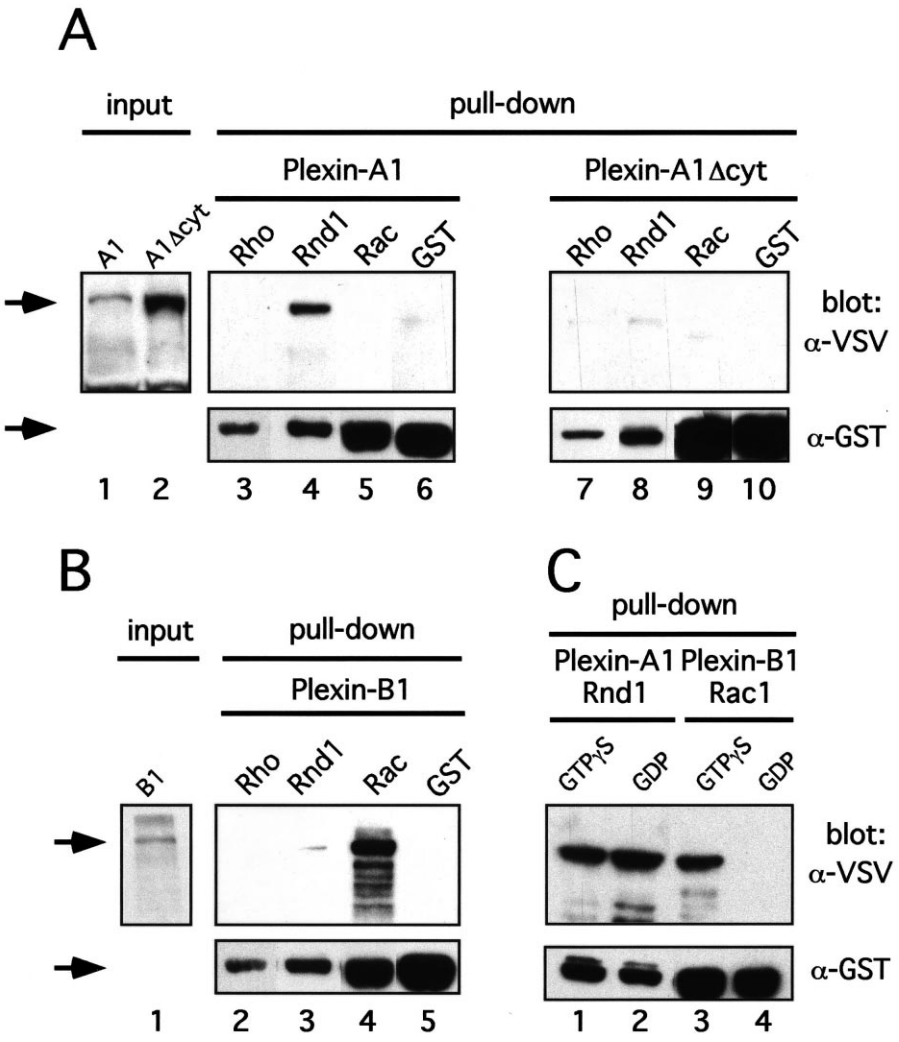

Fig. 2. Interaction between plexins and Rho-like GTPases. A, B: 293T cells were transfected with the expression vectors pBKVSVPlexin-A1 (A: lanes 1, 3–6, C: 1, 2), pBKVSVPlexin-A1Δcyt (A: 2, 7–10), or pBKVSVPlexin-B1 (B: 1–5, C: 3, 4). Cell lysates were incubated with GST-RhoA (A, 3, 7, B: 2), GST-Rnd1 (A: 4, 8, B: 3, C: 1, 2), GST-Rac1 (A: 5, 9, B: 4, C: 3, 4), or GST (A: 6, 10, B: 5) preloaded with GTPγS (A: 3–10, B: 2–5, C: 1, 3) or GDP (C: 2, 4) as indicated and bound proteins analyzed by Western blot using an anti-VSV or anti-GST antibody. Plexin-A1 bound Rnd1, plexin-B1 Rac1 and only weakly Rnd1. C: The interaction between plexin-A1 and Rnd1 was independent of the presence of GPTγS in contrast to that of plexin-B1 and Rac1 that is visible only after loading with GTPγS.

To analyze the consequences of these mutations for the activity of plexin-A1, a heterologous system was employed that mimics growth cone collapse and has been used as a model to study the properties of semaphorin receptors [12]. COS-7 cells became sensitive to Sema3A upon co-transfection with expression vectors for Nrp-1 and plexin-A1 so that addition of Sema3A induced the collapse of 85.4 ± 6.7% (mean ± S.D.) of transfected cells (Fig. 4A,B) resulting in contraction of the cell body that remained connected to the substrate only by retraction fibers. Sema3A had no effect on cells expressing only Nrp-1 (Fig. 4A, and data not shown) [12]. Mutation of either R1429,1430 or R1746 almost completely abolished this effect. Only 8.5 ± 2.2 and 15.5 ± 11.4% of the cells expressing plexin-A1R1, or -A1R2, respectively, responded to addition of Sema3A (Fig. 4A,B), comparable to the effect of the double mutant plexin-A1R12 (6.0 ± 2.1% collapse). Thus, mutation of a single conserved arginine was sufficient to almost completely abolish the ability of plexin-A1 to induce the collapse of COS-7 cells in response to Sema3A.

4. Discussion

Semaphorins have potent and cell-type specific repulsive and attractive effects on a wide variety of axons [3–10,29–32]. Recent work has shown that neuropilins and plexins are essential components of the receptor for class 3 semaphorins with the neuropilins acting as the ligand-binding subunit and plexins as the signal-transducing subunit [12–15]. The cytoplasmic domain of plexin-A1 is essential for its function to mediate repulsion by Sema3A [13–15] and shows sequence similarity to Ras GAPs. In particular, two arginines are conserved between all plexins and several Ras GAPs. The first conserved arginine, R1430, corresponds to the invariant arginine located in the finger-loop of p120 RasGAP that inserts into the active center of Ras [24–26]. The second conserved

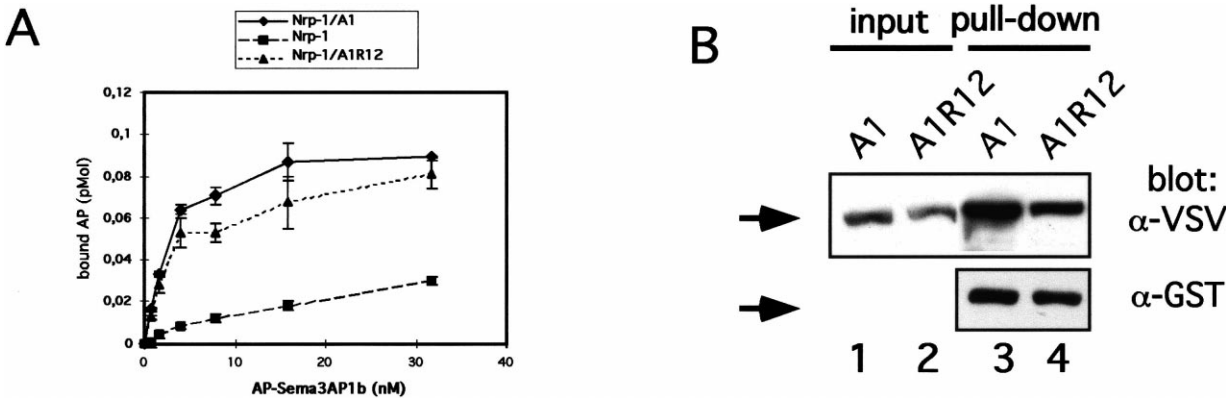

Fig. 3. Mutation of conserved arginine residues in plexin-A1 does not affect interaction with Sema3A or Rnd1. A: 293T cells were transfected with a 1:2 mixture of pBKHANrp-1 and pBK-CMV (broken line), pBKVSVPlexin-A1 (solid line) or pBKVSVPlexin-A1R12 (dotted line) and incubated with increasing concentrations of AP-Sema3AP1b. The amount of bound AP-activity was determined colorimetrically. Plexin-A1 and Plexin-A1R12 did not show a significant difference in their binding properties. B: 293T cells were transfected with pBKVSVPlexin-A1 (lanes 1 and 3) or pBKVSVPlexin-A1R12 (lanes 2 and 4). Cell lysates were incubated with GST-Rnd1 (lanes 3 and 4) and bound proteins analyzed by Western blot using an anti-VSV or anti-GST antibodies. Plexin-A1 and Plexin-A1R12 did not differ in their ability to bind GST-Rnd1.

arginine is involved in stabilizing the finger-loop and the corresponding position in plexin-A1 is occupied by R1746. Mutation of these conserved arginine residues abolished its ability to induce the collapse of COS-7 cells in response to Sema3A.

These data suggest that plexins may directly regulate the activity of small GTPases. Indeed, we could show that the cytoplasmic domain of plexin-A1 binds Rnd1 while plexin-B1 preferentially interacts with Rac1 and less strongly with Rnd1. This interaction was not dependent on the presence of their respective ligands Sema3A and Sema4B or the co-receptor Nrp-1. The differential interaction of two plexins with Rnd1 and Rac1 indicates that, despite the high sequence similarity between the cytoplasmic domains of the plexin family, different types of plexins may activate distinct intracellular signalling pathways. The GTP dependence of its interaction with Rac1 suggests that plexin-B1 is a target for active Rac1 while the binding of GDP–Rnd1 is consistent with a function of plexin-A1 as a GAP. It remains to be shown, however, that Rnd1 indeed is a substrate for a GAP activity of plexin-A1. Rnd1 has low intrinsic GTPase activity and is constitutively GTP-bound [28]. It remained unclear, so far, how the activity of Rnd1 might be regulated and it was suggested that its subcellular localization could play an important role in its function [28,33,34]. Thus, plexins may act to recruit Rnd1 to the plasma membrane and modulate its activity by acting as a GAP.

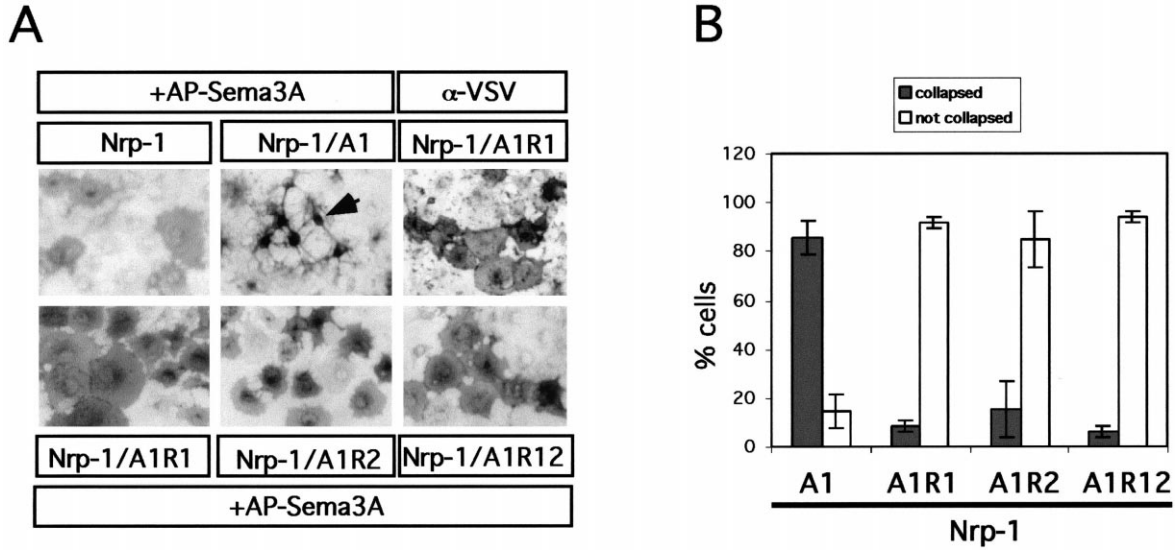

Fig. 4. Mutation of conserved arginine residues inactivates plexin-A1. A: COS-7 cells were transfected with a 1:2 mixture of pBKHANrp-1 (Nrp-1) and pBK-CMV, pBKVSVplexin-A1 (A1), pBKVSVplexin-A1R1 (A1R1), pBKVSVplexin-A1R2 (A1R2) or pBKVSVplexin-A1R12 (A1R12) and incubated with 0.4 nM AP-Sema3A for 1 h at 37°C. Bound fusion proteins were revealed by a histochemical stain for alkaline phosphatase (AP). Cell surface expression of plexin-A1R1 (α-VSV) was analyzed with an AP-coupled secondary antibody confirming that plexin-A1R1 was expressed on the cell surface. The same results were obtained for the other mutants (data not shown). Cells expressing Nrp-1 and plexin-A1 but not those expressing only Nrp-1 collapsed in response to Sema3A (arrow). Mutation of R1429,1430 or R1746 abolished the sensitivity of transfected cells to Sema3A. B: The number of cells collapsed in response to Sema3A was determined according to published criteria [12]. The percentage of collapsed (gray bars) and non-collapsed cells (open bars) is displayed for the different mutants and wild-type plexins ($n = 3$, experiments were done in triplicate and 1200–1800 cells counted per experiment).

In summary, these data suggest that plexins may act as GAPs for Rho- or Ras-related GTPases and directly regulate their activity during growth cone collapse. We propose that activation of the Nrp-1/plexin complex modulates the intrinsic GAP activity of plexins and thereby allows the spatially restricted activation of GTPases, that ultimately results in a depolymerization of actin fibers. This model implies that plexins are the first class of membrane proteins that contain a catalytic domain with GAP activity.

Acknowledgements: We thank N. Büchner for excellent technical assistance, S. Fuchs for help with sequencing, E. Evers for helpful discussions, and C. Burkhardt, R. Fiore, E. Evers and H. Betz for comments on the manuscript. This work was supported by the DFG.

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